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13. ABSTRACT *(Maximum 200 words)*

Wnt/ wingless growth factors cause mammary tumors when overexpressed, and have many developmental functions. Yet despite extensive studies on the Wnt ligands, and the putative pathway components, little is known about the reception mechanisms of signaling by these growth factors.

A candidate Wnt receptor has been isolated using the yeast two-hybrid system. This protein has been shown to specifically bind four Wnt ligands. Truncated receptor forms, when misexpressed in *Drosophila* , lead to wingless loss of function phenotypes, strengthening its legitimacy as a Wnt receptor.

The study's long-term objectives is to further characterize and functionally test this gene product. This will involve studies in both mouse and *Drosophila* , employing both ectopic expression studies and null mutation analysis. The proposed research should verify and elaborate the protein's role in Wnt / wingless signal transduction as it pertains to development and tumorigenesis.

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Mark J. Alves 7/29/98

PI - Signature Date

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Wnt family of signaling molecules

Researchers detected the first member of the *Wnt* family, *int-1*, over a decade ago by its ability to induce mammary tumors (1). Ectopic expression of *int-1* (*Wnt-1*) in mammary tissue by a proviral insertional event induces tumorigenic transformation in adult mice. Since then, many murine *Wnt* family members have been discovered that now comprise the Wnt family of growth factors (2, 3).

Analysis of known *Wnt* proteins has revealed a number of similar features, the principal characteristic being the highly conserved presence, and spacing, of 22 cysteine residues. These residues are critical for *Wnt* function, as determined by site directed mutagenesis in cell transformation assays (4) and for the induction of *Xenopus* axis duplication (5), underscoring the importance of their strong conservation throughout evolution. In addition, each member of the *Wnt* family possesses a hydrophobic signal sequence, presumably for secretion into the extracellular environment, and a highly conserved *N* -linked glycosylation site.

Wnt growth factor family members serve many murine biological roles. *Wnts* RNA localization in different mouse embryonic tissues suggests that Wnt proteins carry out crucial functions in development (6). Indeed, *Wnt* targeted mutations disrupt the development of specific embryonic tissues: midbrain for *Wnt-1* (7, 8), somites caudal to the forelimb for *Wnt-3a* (9) kidney for *Wnt-4* (10), and limb polarity for *Wnt-7a* (11). *Wnt* family members are further implicated in mammary development during pregnancy and lactation (12). Additionally, the appearance of mammary adenocarcinomas in adult mice has been attributed to *Wnt-1* and *Wnt-3* ectopic expression in the affected mammary glands (1, 13).

Wnt growth factors have recently been implicated in human tumor development. Examination of human breast cell lines and tissues have revealed greatly increased *Wnt-2* and *Wnt-4* mRNA levels in fibroadenomas compared to normal tissue (14). Additionally, *Wnt-7b* mRNA levels increased 30 fold in 10% of tumors examined. In a related study, aberrantly high *Wnt-5a* mRNA levels were found in lung, breast, and prostate carcinomas and in melanomas, implicating *Wnt-5a* in the development of these cancers (15).

Wnt family members are also found in *Drosophila* and are critical for many important roles. *wingless* (*Drosophila Wnt-1* homolog) is required throughout embryonic development for epidermis specification, tissue induction, neuroblast differentiation, and Malpighian tubule formation (16). In addition, wingless is responsible in patterning adult structures such as wing, leg, genitalia, eye, abdomen, heart, and brain (17, 18, 19, 20). Finally, there exists at least four non-wingless family members (Dwnts; 21, 22, 23), functions of which have yet to be determined.

Although Wnts/Dwnts are critical for diverse functions, little is known about the molecular mechanisms, or associated proteins, by which Wnt/wingless signal transduction is accomplished. Analysis of *Drosophila* mutant phenotypes, coupled with genetic epistasis experiments (24), have identified candidates in the *wingless* signal transduction pathway (16, 25). However, detecting direct interactions between Wnts and potential signaling has been problematic. To date, the primary difficulty in identifying molecules that interact with Wnt growth factors, such as receptors or extracellular matrix proteins, has been the inability to generate suitable quantities of a soluble, physiological form of a Wnt protein (3). This technical roadblock has hampered more conventional approaches such as expression library screening (26) or expression cloning (27, 28); approaches used successfully to isolate receptors in other systems. Clearly, an alternative approach was required to identify Wnt interacting proteins.

An assay to identify proteins involved in Wnt signaling pathways

The elegant and powerful yeast two-hybrid system has been developed and refined to facilitate the identification of protein-protein interactions (29). A concept first developed by Fields and Song (30), the technique involves constructing a "bait" in which your molecule of choice is fused to a DNA binding domain able to recognize an Upstream Activating Sequence (UAS). A yeast strain, containing a reporter under the control of the UAS, is transformed with the bait. An "activation" library, in which all cDNAs are fused to an activating domain ("prey"), is then transformed into the above strain. Both the bait and the preys have a nuclear localization signal for targeting into the nucleus. The reporter gene will be activated if a cDNA codes for a protein that interacts with the bait (Fig. 1).

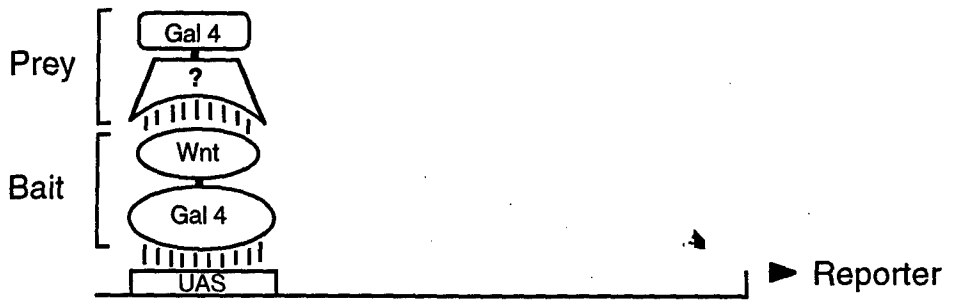

Fig. 1 The yeast two-hybrid system.

Body

I have employed the two-hybrid system in an effort identify proteins that bind Wnt growth factors. Four full-length Wnt "baits" have been constructed; Wnt-1, Wnt-3a, Wnt-4, and Wnt-7b. These baits are a hybrid fusion protein of the Gal4 DNA binding domain and the full-length Wnt protein, minus the putative signal peptide. Activation libraries ("preys") have been obtained and amplified. I have focused on two libraries of embryonic (9.5-10.5 day) and virgin mammary gland origin. Both of these tissues show an aberrant phenotype for either loss of and/or ectopic expression of various *Wnt* genes. Presumably, proteins responsible for receiving Wnt signals are found in these tissues.

To date, I have performed extensive two-hybrid screens with both activation libraries and the four Wnt baits. Positives isolated fall into one of three classes, depicted below in Table 1:

classes	pDAB1	Wnt-1	Wnt-3a	Wnt-4	Wnt-7b	Notch 3	SNF1	CDK2	lamin
I	+	+	+	+	+	+	+	+	+
II	-	+	+	+	+	+	+	+	+
III	-	+	+	+	+	-	-	-	-

Table 1 Interaction types in the two-hybrid system. Classes I-III, on the y-axis, represent 3 prey categories. Test baits are represented on the x-axis. Reporter activation is denoted by a +; reporter inactivation by a -.

Class I

Reporter activation does not necessarily indicate a specific bait and prey interaction. Many false positives (preys) isolated, comprising the first class, activate reporters not only with Wnt baits but also with unrelated control baits and the GAL4 DNA binding domain alone (pDAB1). Obviously these positives are not worth further analysis.

Class II

The positives in this class do not interact with the GAL4 DNA binding domain alone but do bind with all or a subset of control baits. An class II example was a clone coding for 3 EGF repeats found in the murine Notch 3 protein (later reconstructed as a control bait; 31). Although these clones can not be absolutely termed false positives, they are not compelling candidates.

Class III

Molecules in this class are arguably the strongest candidates for Wnt receptors In this case, the isolated prey codes for a protein fragment that binds Wnt baits(s) but not the control baits or the GAL4 DNA binding domain alone. This result does not preclude the class III prey from binding non-Wnt baits but does suggest a certain specificity for Wnts not found in the other two classes. To date, only one clone has been identified in this third class: PPT.

PPT

PPT is a serine/threonine phosphatase originally isolated from both rat (32) and human (33) tissues. Although PPT contains a catalytic domain similar to other known serine/threonine phosphatases, a distinguishing feature is the presence of four tetratricopeptide repeats (TPR) at the N-terminal region. TPR domains are motifs of 34 amino acid that were originally found in yeast proteins involved in mitosis and RNA synthesis (34). Because TPR domains are thought to bind other proteins, it has been speculated that ligand binding to the PPT, TPR domain is responsible for phosphatase activity. The Wnt interacting clone isolated in the two-hybrid screen encodes the TPR domains found in PPT (Fig.2). This clone begins and ends with the four TPR motifs; its identification as part of a phophatase derived from the full-length sequence.

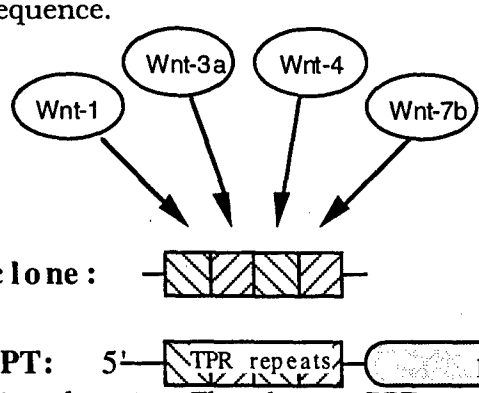

Fig. 2 mPPT interacting domain. The above mPPT protein subfragment, including the TPR motifs, represents the mPPT domain the four Wnt baits initially bound.

Supportive evidence that PPT is a Wnt receptor stems from genetic and biochemical *Drosophila* studies. Two putative downstream components of the *Drosophila wingless* pathway are the protein kinase shaggy (sgg) and armadillo (arm) (35). sgg is believed to down regulate, via phosphorylation, the levels of cytoplasmic arm. It is currently believed that wingless somehow causes inactivation of, or antagonizes, sgg activity. This in turn causes the accumulation of cytoplasmic unphosphorylated arm and subsequent downstream signaling. Studies have revealed this part of the pathway is sensitive to okadaic acid, a phosphatase inhibitor (35). Therefore, sgg-mediated arm regulation may be under phophatase control. PPT (minus the TPR domains) has been shown to possess phophatase activity that is inhibited by okadaic acid (32).

Dominant negative / activated receptor expression.

One approach to analyze receptor function is to suppress a function normally attributed to ligand expression. Overexpression of the receptor's ligand binding domain (lacking the catalytic domain) may effectively bind and sequester free ligand, reducing bound ligand to the endogenous full-length receptor. This might effectively mimic a loss of function ligand mutation and therefore, reveal clues to receptor / ligand function.

Conversely, dominant activated receptor forms often mimic the ligand induced phenotype, irrespective of the ligand's presence or absence (36). This may reproduce gain of function phenotypes obtained by either ligand mutant alleles or ectopic expression studies. Fortunately, truncated forms of PPT may exhibit dominant activation activity. PPT exhibits phosphatase activity when the TPR region is deleted (32).

The putative dominant activating and negative murine PPT (mPPT) forms, in addition to the full-length species, were introduced into *Drosophila* , via P-element-mediated transformation, under the control of the GAL4 activation system (37); Fig. 3]. The GAL4 activation system expresses your choice gene in specific tissues at precise times, depending on the GAL4 driver chosen. Therefore, the GAL4 system allows one to analyze protein function in a variety of tissues at different developmental stages. The assumption in this experiment is that the murine gene will function in *Drosophila* . However, in many cases this can occur; *Wnt /wingless* pathway components have been shown to be functionally conserved between species (38, 39).

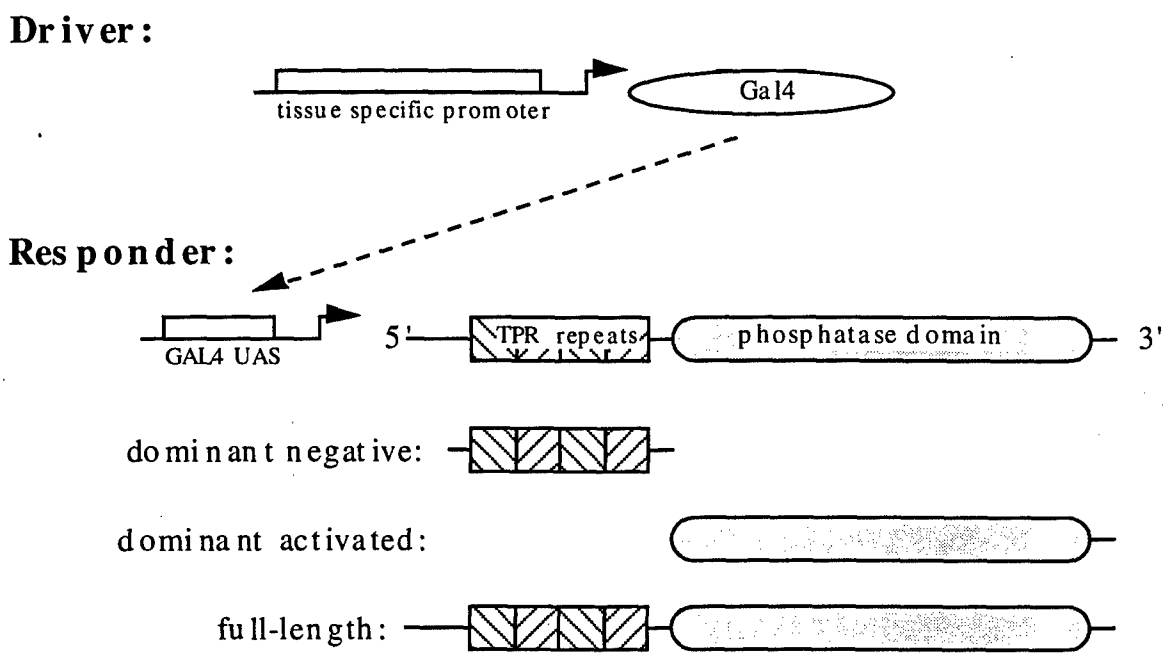

Fig.3 The GAL4 system. The driver construct expresses GAL4 under chosen tissue and temporal conditions. The GAL4 protein in turn activates the responder which then expresses your choice protein in the pre-selected tissues and times.

Recent experiments using the *Drosophila* GAL4 system and the mouse *PPT* gene have revealed a number of interesting phenotypes:

Drivers have been tested that express GAL4 in the *Drosophila* wing imaginal disc; a proven wingless responsive tissue. Different effects are observed, depending on the responder construct type tested and the relative strength of the transposon's expression. For example, the GAL4 imaginal disc driver, line T80 (40), crossed to a weak dominant negative mPPT responder line produces adult flies that exhibit patterning defects in the anterior wing compartment. This is evidenced by the lack of vein 2 and the crossvein connecting veins 3 and 4. In contrast, when another wing disc GAL4 driver, line 71B (37), is tested with the dominant activating responder construct, the distal region and posterior wing compartment shows patterning defects. This is indicated by the loss of the crossvein connecting veins 4 and 5 and severe distal disruptions (data not shown).

Sternites are sensory bristles found on the ventral surface of an adult fly's abdomen which arise from the ventral histoblast nests of the developing larva. By using a wingless temperature sensitive allele, Shirras and Couso had shown sternite development to be under wingless control (19). Loss of functional wingless protein in larval development eliminates sternite formation. When the wing disc GAL4 driver 32B is crossed with a weak dominant negative expressing line, sternites on the adult abdomen of adult flies from this cross are mostly absent. The dominant negative mPPT line crossed to the GAL4 driver 32B closely phenocopies the mutant phenotype obtained by wingless inactivation at late third instar larva stage.

The final ordered array of ommatidia and bristles in the adult eye is the result in part of earlier growth factors interplay in the eye imaginal disc. The morphogenetic furrow, the site of photoreceptor cell differentiation in the disc, begins posteriorly and proceeds anteriorly. This precise initiation site is under wingless control; removal of wingless allows ectopic site initiation (41). Conversely, ectopic wingless expression in the eye disc leads to ommatidia patterning defects and bristle loss. This is consistent with bristle loss observed when wingless is expressed under *sevenless* promoter control (R. Nusse, unpublished report). Interestingly, when the dominant activating mPPT form is expressed under *patched* driver control (40), the adult eyes exhibit a disruption of ommatidia patterning and substantial bristle loss. Consistent with this, the defects localize posteriorly and not anteriorly; consistent because wingless is normally expressed in anterior lateral regions and, therefore, the dominant activated mPPT function may be redundant in this region.

Although the above experiments are preliminary, certain conclusions can be reached:

1) The opposing wing patterning defects observed with the above driver/responder crosses are consistent with a PPT patterning function.

2) The dominant negative responder mimics a loss of wingless function phenotype; sternite absence. Conversely, the dominant activated responder phenocopies a gain of wingless function phenotype in the adult eye. These effects are consistent with PPT having a role in Wnt / wingless signaling.

Isolation of the Drosophila mPPT homolog, dPPT.

Although the above *Drosophila* transgenic experiments are consistent with a PPT role in the Wnt signaling pathway, to further address this component's authenticity, it is important to identify the *Drosophila* homolog. This would offer a plethora of approaches addressing *in-vivo* fly function (see below) and complement murine approaches.

Drosophila wingless signaling pathway components are conserved in vertebrates (16). To isolate the fly mPPT homolog, a low stringency screen was performed with the mPPT, TPR domain and a 3-24 hr. *Drosophila* embryonic cDNA library. One clone, termed dPPT, was identified and isolated which exhibited strong amino acid identity with mPPT. Overall amino acid identity is 59%, similarity is 75%. In the ligand binding and phosphatase domains alone, the % amino acid similarity is 74% and 80%, respectively.

Drosophila dPPT studies

The *Drosophila* gene availability facilitates many experiments which will complement those being pursued in mice.

1- Characterization

dPPT mRNA localization and tissue distribution. Knowledge of the fly dPPT mRNA expression pattern will clarify PPT's Wnt/wingless signaling role. Using the *Drosophila* PPT homolog dPPT as probe, I am performing in situ hybridization on wingless responsive tissues and developmental times. Studies are initially of staged embryos and imaginal discs since wingless receptor upstream (i.e. wingless) and downstream (i.e. dishevelled, armadillo, shaggy, engrailed) component's expression patterns are well characterized (42, 43, 44, 45).

2- Transgenics

The *Drosophila* experiments performed with the mouse PPT homolog yielded highly informative and encouraging results. The approach worked surprisingly well, considering 600 million years of evolution that separate the two species. Nevertheless, it is essential to test the recently isolated fly homolog in *Drosophila* . This may fine tune and reveal subtle interactions that were missed when the related but not identical mouse domains were tested.

dPPT responders, comparable to those built from the mouse gene, have been constructed and transformant fly lines obtained. These are currently being crossed to an extensive array of drivers that express the responder in wingless responsive tissues. The phenotypes will be scored and compared to known wingless gain or loss of function phenotypes. When appropriate, the phenotypes will be complemented by a molecular analysis utilizing pertinent, wingless pathway markers (i.e. engrailed and armadillo RNA and/or antibodies).

3- Null mutant generation

Drosophila wingless null mutations have a distinctive embryonic lethal phenotype, characterized by ectopic denticle growth and patterning. On the molecular level, this is accompanied by armadillo protein loss and reduced engrailed RNA expression in adjacent cells. Wingless signaling pathway components that have been identified by phenocopying wingless nulls include dishevelled, armadillo and engrailed. Conclusive proof of dPPT's role in wingless signaling would be obtained if dPPT nulls produced a morphological and molecular phenotype similar to a wingless null.

A *dPPT Drosophila* mutation will be isolated and analyzed. *dPPT* polytene in situ hybridization will localize where *dPPT* resides. A regional study will be performed to assess available reagents. Depending on the tools, a mutational strategy will be devised and implemented, complementation groups assigned, and dPPT mutation(s) identified. Once obtained, the null mutation will be morphologically and molecularly characterized and its role in wingless signaling assessed.

Mouse mPPT studies

The following ongoing mouse studies address PPT verebrate functions, particularly as it pertains to Wnt signaling.

1- Characterization

a) Embryonic RNA localization and tissue distribution. The Wnt receptor and signaling components are by definition expressed in Wnt responsive tissues. Since Wnts are believed to act locally, evidence of Wnt signaling involvement would be obtained if mPPT expression overlapped the transcript pattern, and mutant phenotype, of the Wnt(s) used as bait. Whole mount mPPT RNA *in situ* hybridization (46) on mouse embryos will be performed to determine RNA presence and localization.

mPPT Northern analysis is being performed including tissues proven or proposed to be Wnt responsive. Wnt responsive tissues included are the HC11 cell line (see below), virgin mammary tissue, and various staged mouse embryos. Tissue analysis further addresses the validity of an interaction and will be useful when planning functional studies.

b) PPT family studies. Vertebrate wingless pathway components are often represented by multiple family members. For instance, *Drosophila dishevelled* has at least three vertebrate homologs (47, 48, 49). The *Drosophila engrailed* gene has a minimum of two vertebrate homologs (50, 51). I will attempt to isolate and study other murine PPT family members. However, given the interaction of PPT with four different Wnts, it is possible that there may be only one *PPT* gene.

2- Null mutant generation

A mutant phenotype often provides strong evidence for a protein's function. A receptor knockout may phenocopy that of the ligand, provided the receptor is not 1) a receptor for other ligands and 2) functionally redundant with other receptors. An example is the kit-ligand (steel) and its receptor, c-kit (52). Alternatively, a signaling pathway component mutant (i.e. receptor) may phenocopy or complement a mutant phenotype of a different component in the same signaling pathway. An example is *Wnt-7a* null inducing limb ventralization (11) and *Engrailed-1* null causing limb dorsalization (50).

The generation of mutations via embryonic stem cell technology is routine in the Bradley laboratory. The mouse genomic locus has been cloned from a 129 Sv/Ev library and mapped to define intron/exon boundaries and restriction sites. A targeting construct was built that replaced the second *mPPT* exon with an hprt cassette. The second *mPPT* exon codes for the ligand binding domain. The mutated *mPPT* allele would only retain approximately 47% of the ligand binding domain. Additionally, if splicing around the hprt cassette occurred, the predicted protein product would be out of frame, eliminating the danger of producing a truncated, aberrantly functional phosphatase.

A *mPPT*-null allele has been created by homologous recombination in embryonic stem cells, with a targeting frequency of approximately 20%, and subsequently introduced into the germ line of chimerae mice (53). Heterozygous mutant mice are currently being bred to produce homozygous mutant nulls. The *mPPT* mutant mice will be analyzed to determine PPT's function and, ultimately, role as it pertains to Wnt signaling.

Conclusion

As with other growth factor families, receptors bind the growth factor and transmit the signal that ultimately results in cell proliferation; normal or abberant. As indicated in the proposal, although some putative components of the *Wnt* pathway(s) exist, virtually nothing is known about the reception mechanisms of the Wnt signals themselves. It is imperative to isolate and study the reception molecule(s) for the Wnt proteins if Wnt-induced oncogenic transformation is to be studied. I believe this study shows much promise in filling in this key piece of the puzzle and will help address how Wnts cause tumorigenesis.

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504 SCOTT STREET
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REPLY TO
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MCMR-RMI-S (70-1y)

1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Encl

DAMD17-94-J-4413	ADB261602
DAMD17-96-1-6112	ADB233138
DAMD17-96-1-6112	ADB241664
DAMD17-96-1-6112	ADB259038
DAMD17-97-1-7084	ADB238008
DAMD17-97-1-7084	ADB251635
DAMD17-97-1-7084	ADB258430
DAMD17-98-1-8069	ADB259879
DAMD17-98-1-8069	ADB259953
DAMD17-97-C-7066	ADB242427
DAMD17-97-C-7066	ADB260252
DAMD17-97-1-7165	ADB249668
DAMD17-97-1-7165	ADB258879
DAMD17-97-1-7153	ADB248345
DAMD17-97-1-7153	ADB258834
DAMD17-96-1-6102	ADB240188
DAMD17-96-1-6102	ADB257406
DAMD17-97-1-7080	ADB240660
DAMD17-97-1-7080	ADB252910
DAMD17-96-1-6295	ADB249407
DAMD17-96-1-6295	ADB259330
DAMD17-96-1-6284	ADB240578
DAMD17-96-1-6284	ADB259036
DAMD17-97-1-7140	ADB251634
DAMD17-97-1-7140	ADB259959
DAMD17-96-1-6066	ADB235510
DAMD17-96-1-6029	ADB259877
DAMD17-96-1-6020	ADB244256
DAMD17-96-1-6023	ADB231769
DAMD17-94-J-4475	ADB258846
DAMD17-99-1-9048	ADB258562
DAMD17-99-1-9035	ADB261532
DAMD17-98-C-8029	ADB261408
DAMD17-97-1-7299	ADB258750
DAMD17-97-1-7060	ADB257715
DAMD17-97-1-7009	ADB252283
DAMD17-96-1-6152	ADB228766
DAMD17-96-1-6146	ADB253635
DAMD17-96-1-6098	ADB239338
DAMD17-94-J-4370	ADB235501
DAMD17-94-J-4360	ADB220023
DAMD17-94-J-4317	ADB222726
DAMD17-94-J-4055	ADB220035
DAMD17-94-J-4112	ADB222127
DAMD17-94-J-4391	ADB219964
DAMD17-94-J-4391	ADB233754